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Highly Specific Targeting of the TMPRSS2/ERG Fusion Gene in Prostate Cancer Using Liposomal Nanotechnology

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14. ABSTRACT

The TMPRSS2/ERG fusion gene occurs in 15-80% of prostate cancer (PCa) lesions. It is absolutely specific for PCa cells, since the fusion transcript is only present in these cells. There is heterogeneity in the structure of the 5' end of the mRNA transcripts of the fusion gene. Some prostate cancers express a single mRNA type, while others express multiple isoforms of the fusion gene that arise via alternative splicing of the initial fusion transcript. We seek to target the four most common and biologically active alternatively spliced fusion gene transcript isoforms using SiRNAs to obtain maximal biological activity in cancers expressing a specific isoform or a combination of isoforms. We propose to use siRNAs specifically targeting the TMPRSS2/ERG mRNA fusion junctions, which are present only in PCa cells, to minimize off-target effects in normal tissues so toxicity should be minimal. Our results support the efficacy of this approach.

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INTRODUCTION

The discovery of recurrent fusion of the androgen-regulated TMPRSS2 gene to the ERG gene in the majority of prostate cancer (PCa) lesions, has led to a paradigm shift in the study of PCa. The TMPRSS2/ERG fusion gene occurs in 15-80% of PCa lesions, depending on the clinical stage, with 40-60% of surgically treated cancers containing the gene fusion. Most studies have shown an association between the presence of the TMPRSS2/ERG fusion and aggressive disease. We have now demonstrated that the TMPRSS2/ERG fusion gene isoforms can enhance proliferation, invasion and motility of prostate epithelial cells. More importantly, knockdown of the fusion gene in a cancer cell line inhibits tumor growth in vivo in an orthotopic mouse model, indicating that the TMPRSS2/ERG fusion gene is a potential therapeutic target which is present in the majority of prostate cancers.

All reports to date indicate that there is significant heterogeneity in the structure of the 5' end of the mRNA transcripts of the fusion gene. Thus, some prostate cancers express a single mRNA type, while others express multiple isoforms of the fusion gene that arise via alternative splicing of the initial fusion transcript. We have characterized 8 fusion types in PCa (1), which have been confirmed by others. In all cases, the fusion mRNA includes the TMPRSS2 exon 1 and often exon 2, as well. The most common transcript contains the TMPRSS2 exon 1 fused to ERG exon 4, such that translation would have to arise from an internal ATG codon and give rise to a slightly truncated protein which we have designated as the Type III isoform. This variant is expressed in 86% of fusion gene expressing prostate cancers, either alone or in combination with other isoforms. Of particular interest is an isoform in which TMPRSS2 exon 2 is fused with ERG exon 4 (designated Type VI). This variant was present in 26% of our cases with fusion gene expression (1). For this isoform, translation can be initiated from the TMPRSS2 translation initiation codon and results in a true fusion protein containing the first five amino acids of the TMPRSS2 gene fused to a slightly truncated ERG protein. We found that expression of this isoform is associated with aggressive disease. Types I and II give rise to full length ERG protein arising from the native ERG ATG and are also associated with more aggressive disease. These isoforms are present in 20% and 11% of fusion gene expressing cancers respectively.

The promise of specific RNA degradation has also generated much excitement for possible use as a novel therapeutic modality. However, in vivo siRNA delivery has proven difficult because of lack of non-toxic and effective systemic delivery methods. We recently developed non-toxic neutrally charged 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC)-based lipsosomal nanovectors (mean size 65nm) that can target siRNA in vivo into tumor cells 10-fold and 30-fold more effectively than cationic lipids and naked siRNA, respectively, leading to significant and robust target gene silencing in orthotopic cancer models.

The TMPRSS2/ERG fusion gene is absolutely specific for prostate cancer cells, since the fusion transcript is only present in these cells. Unfortunately, there is heterogeneity in the structure of the 5' end of the mRNA transcripts of the fusion gene as described above. Thus, some prostate cancers express a single mRNA type, while others express multiple isoforms of the fusion gene that arise via alternative splicing of the initial fusion transcript. We seek to target the four most common and biologically active alternatively spliced fusion gene transcript isoforms, which constitute greater than 95% of all transcripts, to obtain maximal biological activity in cancers expressing a specific isoform or a combination of isoforms. In vivo knockdown of TMPRSS2/ERG fusion gene expression using liposomal nanovectors should decrease prostate cancer progression in vivo and be an effective therapeutic strategy in human prostate cancers bearing this fusion gene. Given the extremely high prevalence of this chromosomal alteration in human prostate cancer, the majority of prostate cancers may be amenable to this treatment. We propose to use siRNAs specifically targeting the TMPRSS2/ERG mRNA fusion junctions, which are present only in PCa cells, to minimize off-target effects in normal tissues so toxicity should be minimal.

BODY

As outlined in our Statement of Work a number of tasks were proposed for the first 12 months; most of these tasks have been accomplished. For the sake of clarity these tasks will be grouped under three main goals.

Goal 1. Evaluation of the efficacy of the SiRNA knockdown of the Type III fusion gene in vivo

Results:

We designed as series of 18 siRNAs spanning the fusion junction of the TMPRSS2 and ERG genes in the Type III fusion mRNA. We then tested these SiRNAs systematically using transient transfection in 293T, PNT1a expressing the Type III fusion gene and VCaP cells using Western blot and/or quantitative RT-PCR. Of the 18 original SiRNAs we identified three that gave strong, consistent and reproducible knockdown of the Type III TMPRSS2/ERG fusion gene. Figure 1 shows a Western blot of with anti-V5 antibody on cell extracts of 293T cells transiently transfected with V5-Tagged Type III fusion gene and several SiRNAs. Control cells are liposomes only while scrambled represents a non-specific SiRNA. As can be seen in Figure 1, Si8, Si11 and Si14 all give very strong knockdown of the fusion gene. These results were confirmed by quantitative RT-PCR in 293T, PNT1a with Type III fusion and VCaP cells. Based on these results we moved forward with our in vivo experiments using DOPC liposomes to deliver Si8 and Si14 in an orthotopic VCaP model. This experiment is outlined in Figure 2. One week after orthotopic injection mice with luciferase-expressing VCaP cells, treatment was initiated with SiRNAs delivered using DOPC liposomes. Mice were injected with control or twice weekly. Mouse weight was followed and tumor imaging was performed weekly using a Xenogen imaging system after luciferin injection. The experiment was terminated after 4 weeks of treatment and primary tumors weighed and submitted for histopathology and complete necropsy performed on mice. Mice were euthanized 48 hours following the last injection of SiRNA. Of note, no toxicity was noted in any mouse. Tumor weights are shown in Figure 3. Both the Si8 and Si14 groups showed a significant decrease in tumor weight ($p<.001$, t-test) when compared to scrambled control. Luciferase imaging was concordant with the final tumor weight ($r^2=.649$, $p<.0001$). Both SiRNAs decreased tumor weight by approximately 50%. We have begun analysis of tumors from all three groups. Our initial quantitative RT-PCR results indicate a 37% knockdown of the fusion gene in Si8 treated mice and a 40% knockdown in Si14 treated mice. These results indicate that we will need to increase SiRNA delivery in order to more effectively inhibit tumor growth. Analysis of tumors using Ki-67 and CD-31 immunohistochemistry and TUNEL is in progress.

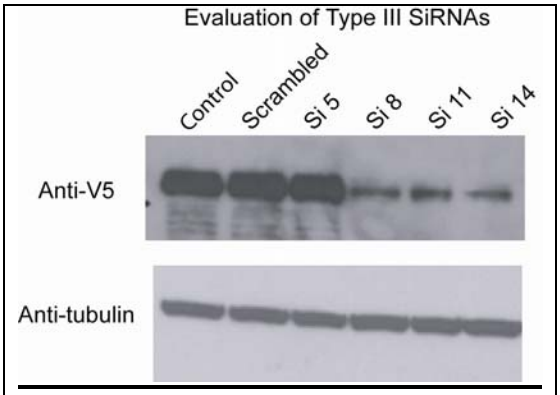

Fig 1. Western blot with anti-V5 antibody of 293T cells transfected with V5-tagged Type III fusion gene, liposomes only (control), scrambled SiRNA and four targeting SiRNAs. Tubulin is a loading control.

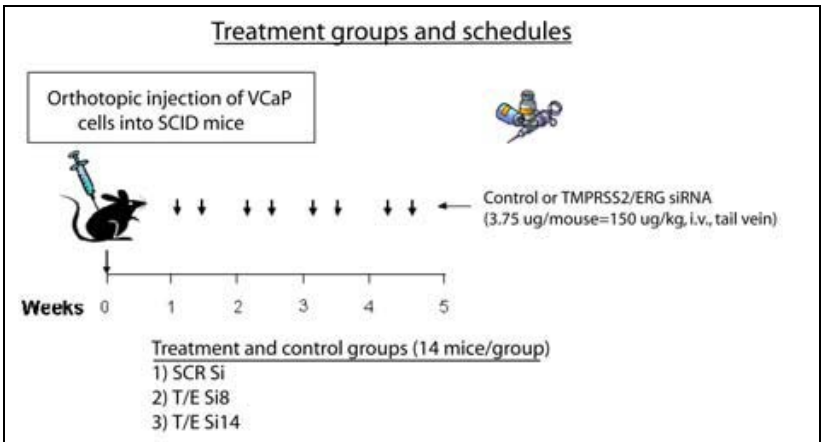

Fig 2. Treatment plan for mice following orthotopic injection of VCaP using fusion specific SiRNAs.
14 mice were used per group

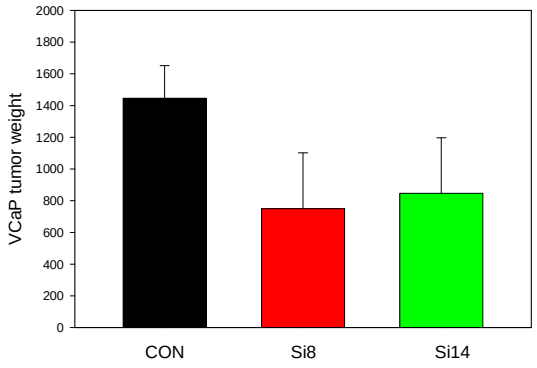

Fig 3. Tumor weight after treatment with Type III fusion mRNA targeting SiRNAs.

Tumor weight at the termination of the experiment outlined in Figure 2 is shown. Mean +/- SD.

Specific Tasks related to this goal.

Months 1-2

1. Submit animal protocols and obtain approvals
2. Design and obtain siRNAs for Type III isoform and begin in vitro testing
3. Establish VCaP cells expressing V5-tagged Type III fusion isoform and V5-tagged ERG

Months 2-5

1. Evaluate Type III candidate siRNAs for knockdown efficacy of fusion gene by Western blot and quantitative RT-PCR using PNT1a cell lines expressing Type III isoform and control cell lines (V5-tagged wild type ERG and TMPRSS2 or control vector transfected cells). The goal is to obtain a siRNA targeting a junctional sequence which will knockdown the fusion gene by 90-95% (at the protein and/or mRNA level) without affecting wild type ERG or TMPRSS2.
2. Evaluate most effective candidate Type III candidate siRNAs for knockdown efficacy of fusion gene by Western blot and quantitative RT-PCR using VCaP cell lines expressing V5-tagged Type III isoform and control cell lines (V5-tagged wild type ERG and TMPRSS2 or control vector transfected cells).

Months 6-9

1. Evaluate in vivo efficacy of best candidate Type III fusion gene specific siRNA by treatment of mice bearing VCaP orthotopic tumors with siRNA incorporated into DOPC liposomes and controls (total of 80 mice; see Proposal). Observe mice for non-specific toxicities during treatment. Euthanize mice after 3 weeks of treatment and weigh and collect snap frozen and formalin fixed tumor and perform full necropsy.
2. Perform histopathological analysis of tumors and all organs from mice (Ittmann)
3. Perform Ki-67 and CD31 immunohistochemistry and TUNEL on all tumors and quantitate.
4. Evaluate expression of fusion gene and total ERG by Western blotting and quantitative RT-PCR using protein extracts and RNAs from tumors.

Goal 2: Evaluation of the efficacy of the SiRNA knockdown of the Type VI fusion gene in vivo

Results:

We designed as series of 18 siRNAs spanning the fusion junction of the TMPRSS2 and ERG genes in the Type VI fusion mRNA. We then tested these SiRNAs systematically using transient transfection in 293T, PNT1a expressing the Type VI fusion using Western blot and/or quantitative RT-PCR. Of the 18 original SiRNAs we identified four that gave strong, consistent and reproducible knockdown of the Type VI TMPRSS2/ERG fusion gene. Figure 4 shows a Western blot of with anti-V5 antibody on cell extracts of 293T cells transiently transfected with V5-Tagged Type VI fusion gene and several SiRNAs. Control cells are liposomes only while scrambled represents a non-specific SiRNA. As can be seen in Figure 4, Si1, Si8, Si14 and Si15 all give very strong knockdown of the fusion gene. These results were confirmed by quantitative RT-PCR in 293T and PNT1a with Type VI fusion.

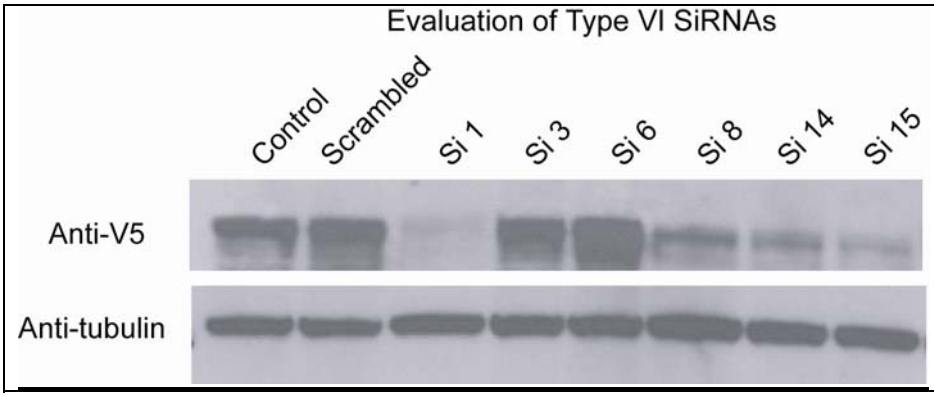

Fig 4. Western blot with anti-V5 antibody of 293T cells transfected with V5-tagged Type III fusion gene, liposomes only (control), scrambled SiRNA and six targeting SiRNAs. Tubulin is a loading control.

We are in the process of establishing VCaP cell lines expressing Type I, Type II, Type III and Type VI fusion gene isoforms. We initially established Type III and Type VI overexpressing cells but unfortunately the Type VI cells were contaminated as we were growing them out so we have initiated new cells so that all cells will be similar passage number when the in vivo experiments are initiated. When these cell lines are ready we plan to initiate in vivo experiments using the Type VI SiRNAs Si1 and Si15.

Specific Tasks related to this goal.

Months 1-2

1. Submit animal protocols and obtain approvals

Months 6-9

1. Establish VCaP cell lines expressing V5-tagged Type I, II and VI fusion isoforms.
2. Design and obtain siRNAs for Type VI isoform

Months 10-12

1. Evaluate Type VI candidate siRNAs for knockdown efficacy of fusion gene by Western blot and quantitative RT-PCR using PNT1a cell lines expressing Type VI isoform and control cell lines (V5-tagged wild type ERG and TMPRSS2 or control vector transfected cells). The goal is to obtain a siRNA targeting a junctional sequence which will knockdown the fusion gene by 90-95% (at the protein and/or mRNA level) without affecting wild type ERG or TMPRSS2.
2. Evaluate the most effective Type VI candidate siRNAs for knockdown efficacy of fusion gene by Western blot and quantitative RT-PCR using VCaP cell lines expressing V5-tagged Type VI isoform and control cell lines (V5-tagged wild type ERG and TMPRSS2 or control vector transfected cells).

Goals for next 12 months

We are encouraged by our ability to find potent SiRNAs targeting both the Type III and Type VI fusions within the limited sequence encoded by the each specific fusion junction. In addition, the ability to inhibit tumor growth in vivo without toxicity using the Type III SiRNAs is encouraging. However our goal is inhibit tumor growth more potently and based on our initial quantitative RT-PCR data we believe we will need to deliver more SiRNA to the tumors to achieve this goal. In our first in vivo study we have used 3.75 ug per injection in DOPC liposomes. To increase SiRNA delivery we plan to increase the SiRNA dose to 10 ug per injection. This dose has been well tolerated in studies using a subcutaneous melanoma xenograft model (2). This should significantly enhance fusion gene knockdown. We will finish the tumor analysis for both of these studies as well.

We will initiate the studies with the Type VI targeting SiRNAs once we have determined whether the 10ug per injection dose is more potent in the Type III SiRNA experiments outlined above. After completing these studies we will characterize the tumor responses to SiRNA treatment.

Finally, we have already initiated design of SiRNAs for the Type I and II fusion mRNAs and the derivation of the appropriate cell lines to analyze the efficacy of these SiRNAs

KEY RESEARCH ACCOMPLISHMENTS

- Developed high efficiency SiRNAs targeting the Type III fusion gene mRNA.
- Developed high efficiency SiRNAs targeting the Type VI fusion mRNA.
- Showed efficacy and lack of toxicity in vivo of SiRNAs delivered via DOPC liposomes using an orthotopic VCAP model.

REPORTABLE OUTCOMES

- Identification of junction specific SiRNAs targeting the most common isoforms of the TMPRSS2/ERG fusion gene
- Using DOPC liposomes to deliver specific SiRNAs targeting the Type III fusion gene isoform we have demonstrated statistically significant downregulation of tumor progression in vivo.

CONCLUSION

Our preliminary results strongly support the concept that we can specifically target the TMPRSS2/ERG fusion gene in vivo using SiRNAs. We will need to further optimize this system to maximize potential therapeutic benefit.

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